Dipeptide Phosphonates as Inhibitors of Dipeptidyl Peptidase IV

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A series of dipeptides which contained phosphonate analogs of proline and piperidine-2-carboxylic acid (homoproline) have been synthesized and tested as inhibitors of DPP-IV. The rates of inhibition of DPP-IV by these compounds are moderate, but the inhibitors are quite specific. The best inhibitor in the series is Ala-Pip^P(OPh-4-Cl)$_2$ (**13**), which has a k_{inact} of 0.353 s^{-1} and K_I of 236 μM. The DPP-IV inhibitors Ala-Pro^P(OPh)$_2$ (**6**), Ala-Pro^P(OPh-4-Cl)$_2$ (**12**), and Ala-Pip^P(OPh-4-Cl)$_2$ (**13**) do not inhibit trypsin, human leukocyte elastase (HLE), porcine pancreatic elastase (PPE), acetylcholinesterase, papain, and cathepsin B. However, compounds **12** and **13** inhibited chymotrypsin slowly. Most of these dipeptides containing a homoproline phosphonate residue (Pip^P) or a Pro phosphonate residue (Pro^P) at the P$_1$ site are stable in a pH 7.8 buffer with half-lives of several hours to several days. DPP-IV inhibited by **6**, **7** (Ala-Pip^P(OPh)$_2$), **12**, or **13** is quite stable, and no enzyme activity was recovered after removal of excess inhibitor and incubation in buffer for 1 day. Since the phosphonate inhibitors are specific toward DPP-IV and the inhibited enzymes are stable, they should be useful in establishing the biological functions of DPP-IV and may be useful therapeutically in the prevention of the rejection of transplanted tissue.

Introduction

Dipeptidyl peptidase IV (DPP-IV,[1] EC 3.4.14.5, CD26) is a post-proline cleaving enzyme which will remove the dipeptides AA-Pro (AA = amino acid residue) from the N-terminus of proteins or polypeptides. DPP-IV has been found in a variety of mammalian cells and tissues, including kidney, placenta, and blood plasma, and on the surface of certain T-lymphocyte subsets. Despite extensive studies, the biological role of DPP-IV in mammalian systems have not been established, although a number of functions have been postulated. DPP-IV may participate in the metabolism and uptake of proline-containing peptides in the intestine and kidney[2] and may be involved in fibronectin-mediated cell movement and adhesion.[3] DPP-IV may also play a role in the metabolism or catabolism of collagen which has a high frequency of Gly-Pro sequences.[4] DPP-IV in human plasma has been shown to cleave N-terminal Tyr-Ala from growth hormone-releasing factor and cause inactivation of this hormone.[5] DPP-IV is also involved in T-cell activation and regulation of T-cell proliferation.[6] Thus, inhibitors of DPP-IV may have therapeutic utility in the modulation of the rejection of transplanted tissue by the host organism.

DPP-IV is a serine protease which has been demonstrated by its complete inhibition by DFP.[7] The amino acid sequence of rat liver DPP-IV deduced from cDNA has been established, and it contains the sequence of Gly-Trp-Ser-Tyr-Gly corresponding to the common sequence Gly-X-Ser-X-Gly found in the active site of various serine proteases.[8] Recent studies have shown that radiolabeled [^{3}H]DFP is bound to Ser-631 of DPP-IV, and the residues Gly-629, Ser-631, and Gly-633 are essential for the enzyme activity of DPP-IV.[9] Interestingly, the catalytic triad residues (Ser-624, Asp-702, and His-734) of mouse DPP-IV are arranged in a novel sequential order (e.g., Ser-Asp-His) that is different from that of archetypical serine proteases (His-Asp-Ser).[10]

A wide variety of inhibitors of serine proteases have been reported;[11] however, only a few classes of compounds are effective inhibitors of DPP-IV. *N*-Peptidyl-*O*-(4-nitrobenzoyl)hydroxylamines irreversibly inactivate DPP-IV, but most of the inhibitor is hydrolyzed during the inhibition process.[12] Azapeptides such as Ala-AzaPro-OPh form acyl enzyme derivatives with DPP-IV, but they are not very effective inhibitors, and the acyl enzyme can deacylate regenerating active enzyme.[13] The boronic acids Ala-boroPro and Pro-boroPro are specific and potent reversible inhibitors of DPP-IV with K_I values in the nanomolar range; however, both inhibitors are unstable in solution at neutral pH.[14] Many other types of transition-state inhibitors would be expected to be quite unstable when incorporated into a dipeptide structure with a free N-terminal amino group. For example, dipeptide trifluoromethyl ketones, α-keto acids, or chloromethyl ketones would be expected to cyclize and be unstable in aqueous solution.[15] Therefore, new specific and potent inhibitors of DPP-IV are needed.

A variety of peptide phosphorus derivatives have been reported to be serine protease inhibitors. For example, DFP analogs such as peptide phosphonyl fluorides inhibit serine proteases potently, but they are extremely unstable in aqueous solution.[16] Peptide phosphonates which incorporate a tetrahedral phosphorus moiety in the peptide substrate inhibit serine proteases poorly.[17] Peptidyl (α-aminoalkyl)phosphonate diphenyl esters offer one potential route to effective DPP-IV inhibitors since phosphonate esters are relative unreactive with

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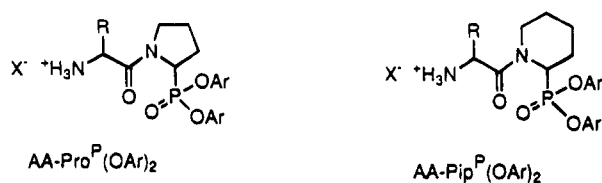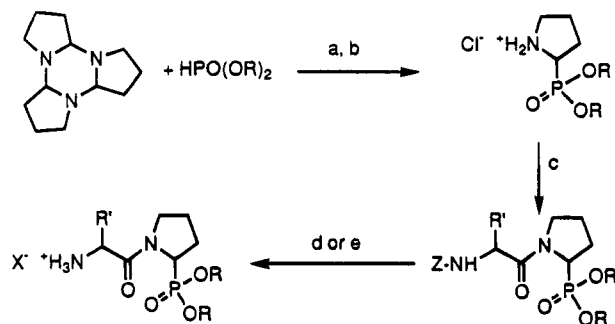§ Current address: OsteoArthritis Sciences, Inc., One Kendall

Figure 1. Structures of Pro- or Pip-containing dipeptide phosphonates, where R is the side chain of Ala, Phe, or Lys and Ar is phenyl, 4-chlorophenyl, or 4-fluorophenyl.

Scheme 1[a]

[a] Reagents: (a) heat under argon; (b) HCl gas in ether; (c) Z-NHCHR'COOH, DCC; (d) Pd/C, H_2, H^+; (e) 30% HBr/AcOH.

potent inhibitors of several serine proteases including PPE, HLE, and chymotrypsin.[18] A good interaction with the S_1 pocket[19] of the enzyme is required for inactivation of serine proteases by these peptide phosphonates, and the interaction with extended substrate binding site of the enzyme is also essential for effective inhibition. In this paper we report the syntheses of several dipeptide phosphonates and their inhibitory activities against DPP-IV and other proteases.

Results and Discussion

Chemistry. A series of dipeptides which contain 2-pyrrolidylphosphonate (proline phosphonate, Pro^P) or 2-piperidylphosphonate (homoproline phosphonate, Pip^P) (Figure 1) were synthesized using the reactions outlined in Scheme 1. The proline phosphonate HCl·$Pro^P(OR)_2$ was synthesized by reaction of diphenyl phosphite or bis(4-chlorophenyl) phosphite with 1-pyrroline trimer.[20] Subsequent coupling of HCl·$Pro^P(OPh)_2$ (1) or HCl·$Pro^P(OPh-4-Cl)_2$ (3) with the N-blocked amino acid Z-AA-OH using the DCC method gave the dipeptide phosphonate Z-AA-$Pro^P(OR)_2$. Deblocking of the dipeptides was accomplished by hydrogenolysis in the presence of acid or by the use of 30% HBr in AcOH to give compounds 6, 9, 10, and 12. Similarly, the homoproline derivative HCl·$Pip^P(OPh)_2$ (2), HCl·$Pip^P(OPh-4-Cl)_2$ (4), or HCl·$Pip^P(OPh-4-F)_2$ (5) was synthesized by reaction of diphenyl phosphite or bis(4-halophenyl) phosphite with 2,3,4,5-tetrahydropyridine trimer.[21] The intermediate $Pip^P(OR)_2$·HCl was then coupled with Z-AA-OH using the DCC method to give the dipeptides Z-AA-Pip^P-$(OR)_2$. Subsequent deblocking of Z-AA-$Pip^P(OR)_2$ with hydrogenolysis in the presence of acid or HBr in AcOH gave compounds 7, 12, 13, and 14. These dipeptide phosphonates were prepared as mixtures of diastereomers. Silica gel column chromatography and preparative thin-layer chromatography were used to attempt the separation of these two isomers. In most cases, the

Table 1. Inhibition of Human Placenta DPP-IV by Peptidyl Phosphonates[a]

| | inhibitors | [I] (mM) | % inhibition | |
			2 min	30 min
6	HCl·Ala-$Pro^P(OPh)_2$	0.12	0	33
7	AcOH·Ala-$Pip^P(OPh)_2$	0.12	0	100
8	AcOH·Ala-$Pip^P(OH)(OPh)$	0.12	0	0
9	HBr·Phe-$Pro^P(OPh)_2$	0.12	0	0
10	2HBr·Lys-$Pro^P(OPh)_2$	0.12	0	0
11	2HCl·Lys-$Pip^P(OPh)_2$	0.12	35	88
12	HCl·Ala-$Pro^P(OPh-4-Cl)_2$	0.12	0	100
13	HCl·Ala-$Pip^P(OPh-4-Cl)_2$	0.12	88	100

[a] Percentage inhibition was measured after 2 or 30 min incubation in 0.05 M Tris, pH 7.8 buffer and 5% Me_2SO and at 23 °C. TFA·Ala-Pro-AFC (0.190 mM) was used as the substrate.

Table 2. Rates of Inhibition of DPP-IV by Peptide Phosphonates and Half-Lives for Hydrolysis of Peptide Phosphonates[a]

	inhibitors	$t_{1/2}$ (h)	[I] (mM)	$k_{obs}/[I]$ $(M^{-1} s^{-1})$
6	HCl·Ala-$Pro^P(OPh)_2$	23.1	0.42	1.2
7	AcOH·Ala-$Pip^P(OPh)_2$	>72	0.42	12.6
10	2HBr·Lys-$Pro^P(OPh)_2$	52	0.42	1.7
11	2HCl·Lys-$Pip^P(OPh)_2$	>48	0.42	4.2
12	HCl·Ala-$Pro^P(OPh-4-Cl)_2$	5.3	0.42	28
13	AcOH·Ala-$Pip^P(OPh-4-Cl)_2$			
	(diastereomeric mixture)	67	0.017	156
	(single diastereomer)	>48[b]	0.017	1300
14	HCl·Ala-$Pip^P(OPh-4-F)_2$	>68	0.42	12

[a] Hydrolysis and inhibition were measured in 0.05 M Tris, pH 7.8 buffer and 8% Me_2SO and at 23 °C. TFA·Ala-Pro-AFC (0.2 mM) was used as the substrate. [b] ^{31}P NMR spectra were used to monitor the hydrolysis.

peaks with different ratios in the ^{31}P NMR spectra of the phosphonates. Only one compound (13, CH_3-COOH·Ala-$Pip^P(OPh-4-Cl)_2$) showed one peak in the ^{31}P NMR spectrum after chromatography. It is likely that this isomer is L,L (see Kinetics section), although we cannot exclude the possibility that both diastereomers are present.

Inhibition Kinetics. The results of initial inhibition studies of DPP-IV by a series of dipeptide phosphonates are shown in Table 1. At inhibitor concentrations of 0.12 mM and with a 2 min incubation time, only Lys-$Pip^P(OPh)_2$ (11) and Ala-$Pip^P(OPh-4-Cl)_2$ (13) effectively inhibited DPP-IV. With a 30 min incubation time, five dipeptide phosphonates (6, 7, 11, 12, 13) showed some inhibitory potency. The monoester Ala-$Pip^P(OH)(OPh)$ (8), compound 9 with Phe at the P_2 site, and 10 did not show any inhibition of DPP-IV under these conditions.

The second-order inhibition rate constants $k_{obs}/[I]$ for the better inhibitors are shown in Table 2. All the phosphonates in Table 2 are mixtures of both diastereomers. Compound 13 was also initially obtained as a mixture of diastereomers. Upon further chromatography, one diastereomer (probably L,L) was obtained which showed one peak in the ^{31}P NMR spectrum.

The best inhibitor in the series is Ala-$Pip^P(OPh-4-Cl)_2$ (13) which has a k_{obs} value of 1300 M^{-1} s^{-1}. The k_{inact} (0.353 s^{-1}) and K_I (236 μM) values of this compound are obtained from Kitz and Wilson plot [$k_{obs} = k_{inact}[I]/(K_I + [I])$].[22] The inhibitory potency of the single diastereomer of 13 was 8-fold higher than the diastereomer mixtures. Substitution of phenoxy by a 4-chlorophenoxy group improves the inhibition rate by 12–23-fold (12 > 6; 13 > 7). However, the inhibition rate of DPP-IV by

Table 3. Inhibition of Proteases and Esterases by Dipeptide Phosphonates[a]

| | $k_{obs}/[I]$ (M^{-1} s^{-1}) | | |
enzymes	Ala-Pro^P(OPh)$_2$ (6)	Ala-Pro^P(OPh-4-Cl)$_2$ (12)	Ala-Pip^P(OPh-4-Cl)$_2$ (13)
chymotrypsin	NI[b]	26	18
trypsin	NI	NI	NI
HLE	NI	NI	NI
PPE	NI	NI	NI
acetylcholinesterase	NI	NI	NI
papain	NI	NI	NI
cathepsin B	NI	NI	NI

[a] Inhibition was measured in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer (chymotrypsin, PPE, HLE), 0.1 M Hepes, 0.01 M CaCl$_2$, pH 7.5 (trypsin), 0.1 M phosphate, pH 7.5 (acetylcholinesterase), 0.05 M Tris, 2 mM EDTA, 5 mM cysteine, pH 7.5 (papain), or 0.1 M phosphate, 1.33 mM EDTA, 2.7 mM cysteine, pH 6.0 (cathepsin B), 8−9% Me$_2$SO and at 23 °C. Substrates were Suc-Phe-Thr-Phe-pNA (0.48 mM) for chymotrypsin, Z-Phe-Gly-Arg-pNA (0.09 mM) for trypsin, MeO-Suc-Ala-Ala-Pro-Val-pNA (0.24 mM) for HLE, Suc-Ala-Ala-Ala-pNA (0.44 mM) for PPE. Inhibitor concentrations were 0.42 mM. [b] NI, no inhibition after 30 min of incubation of enzyme with inhibitor.
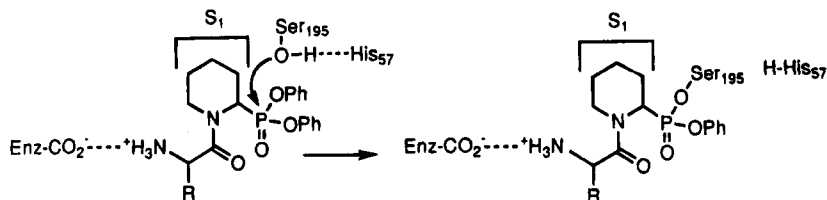

Figure 2. Inhibition mechanism of DPP-IV by the dipeptide phosphonate, Ala-Pip^P(OPh)$_2$. The mechanism involves the nucleophilic substitution at the phosphorus atom by the active site Ser-195 through a pentavalent intermediate to form a phosphonylated enzyme.

phenoxy derivative **7**. Replacing the Pro phosphonate by a homoproline phosphonate (Pip^P) also enhanced the inhibition by 2−10-fold (**7** > **6**; **11** > **10**; **13** > **12**). Previous studies with synthetic substrates demonstrated that DPP-IV hydrolyzed the dipeptide p-nitroanilides AA-Pro-pNA faster when the P$_2$ site contained a Pro, Abu, Leu, Val, or Ala rather than Phe or Lys.[23] In the Pro or homoproline-containing phosphonate inhibitors, Ala is preferred at the P$_2$ site rather than Lys or Phe. For example, Ala-Pro^P(OPh)$_2$ (**6**) but not Phe-Pro^P(OPh)$_2$ (**9**) or Lys-Pro^P(OPh)$_2$ (**10**) inhibited DPP-IV at 0.12 mM and 30 min incubation (Table 1). Similarly, Ala-Pip^P(OPh)$_2$ (**11**) inhibited DPP-IV more potently than Lys-Pip^P(OPh)$_2$ (**7**). Interestingly, both Pro and homoproline-containing dipeptide phosphonates inhibited DPP-IV and the substitution of a Pro phosphonate by a homoproline phosphonate enhanced the inhibition rates. This indicates that the S$_1$ pocket of DPP-IV is bigger than a proline ring and can accommodate the larger homoproline structure.

The specificity of these dipeptide phosphonates for DPP-IV was examined by measuring inhibition rates with other proteases and esterases. The results were shown in Table 3. Three inhibitors, Ala-Pro^P(OPh)$_2$ (**6**), Ala-Pro^P(OPh-4-Cl)$_2$ (**12**), and Ala-Pip^P(OPh-4-Cl)$_2$ (**13**), inhibited DPP-IV but not six other proteases and esterases. Two chlorophenoxy phosphonates **12** and **13** inhibited chymotrypsin very slowly, which is surprising since chymotrypsin does not hydrolyze peptide substrates with Pro at the P$_1$ site. We postulate that one of the two 4-chlorophenoxy groups in inhibitors **12** and **13** is fitting into the large hydrophobic S$_1$ site of chymotrypsin, and this result shows in the inhibition rates. With this exception, the inhibitors are highly specific for DPP-IV.

Spontaneous Hydrolysis of Dipeptide Phosphonates. Peptide phosphonates are known to be stable

inhibitors are quite stable with half-lives of several hours to several days. Phosphonates with homoproline at the P$_1$ site are more stable than those with Pro ($t_{1/2}$: **7** > **6**; **13** > **12**). The ^{31}P NMR spectra were used to monitor the hydrolysis of compound **13** in 50 mM Tris, pH 7.8 buffer containing 10% DMSO. The spectra show only one peak at 19.8210 ppm initially, and no extra peaks appear during a period of 48 h. This result indicates that the inhibitor is stable in the pH 7.8 buffer and does not react with Tris as in the earlier studies with different phosphonates under different conditions.[24] The inhibition of DPP-IV by CH$_3$COOH·Ala-Pip^P(OPh-4-Cl)$_2$ went to completion in a few min giving a stable phosphonylated enzyme ($t_{1/2}$ >24 h). Thus, no reaction of the phosphonylated enzyme with Tris occurred in our case. The ^{31}P NMR also indicates the cyclization of the free amino group onto the phosphorus does not occur at this pH. Phosphonate are relatively unreactive to nitrogen nucleophiles and the amine group is protonated at this pH.

Inhibition Mechanism. The proposed inhibition mechanism of DPP-IV by the dipeptide phosphonate Ala-Pip^P(OPh)$_2$ (**7**) is similar to that previously described for other serine proteases (Figure 2).[18] It involves the nucleophilic substition at the phosphorus atom by the active site Ser-195 through a pentavalent intermediate to form a phophonylated enzyme. The leaving group in these dipeptide phosphonates is an electronegative phenoxy or 4-halophenoxy group. The Pro or Pip residue fits into the S$_1$ pocket. The DPP-IV which was inhibited by compounds **6**, **7**, **12**, or **13** was stable and did not regain enzyme activity after 24 h. Excess inhibitors in the inhibited enzyme solution were removed by centrifugation of the diluted enzyme solution twice using Amicon microconcentrators. These results are consistent with the formation of a stable phosphonylated enzyme derivative. A similar mechanism has

phosphonate where *m*-hydroxybenzoate was released stoichiometrically.[25] The phosphonylated enzymes have also been observed in the crystal structures of α-lytic protease with two stereoisomers of Boc-Ala-Ala-Pro-Val^P(OPh)-Lac-Ala-OMe.[24,26] In the complex of one isomer, the phenyl ester is displaced by the active site serine to form a tetrahedral adduct. In the complex of the other isomer, the same tetrahedral adduct and an adduct with both ester groups hydrolyzed were observed.

Conclusion

A series of dipeptide phosphonates which contained a Pro or a homoproline analog (Pip^P) at the P$_1$ site are specific irreversible inhibitors of DPP-IV. The dipeptide phosphonates are moderate inhibitors of DPP-IV with k_{obs}/[I] values of 1–1300 M^{-1} s^{-1} and the best inhibitor is Ala-Pip^P(OPh-4-Cl)$_2$ (**13**). Since the 4-chlorophenoxy group is a better leaving group than the phenoxy substituent, it is expected that the dipeptide phosphonates with 4-chlorophenoxy groups would hydrolyze faster in buffer and inhibit DPP-IV more potently than those with phenoxy groups. The phosphonates are quite specific, and Ala-Pro^P(OPh)$_2$ (**6**), Ala-Pro^P(OPh-4-Cl)$_2$ (**12**), and Ala-Pip^P(OPh-4-Cl)$_2$ (**13**) did not inhibit proteases and esterases such as trypsin, HLE, PPE, acetylcholinesterase, papain, and cathepsin B, although **12** and **13** inhibited chymotrypsin fair slowly. Most of these dipeptide phosphonates are stable in pH 7.8 buffer with half-lives of several hours to several days. DPP-IV inhibited by **6**, **7**, **12**, or **13** is quite stable, and no enzyme activity was regained after removal of excess inhibitors and incubation in the buffer for 1 day. Due to their high specificity and stability, these dipeptide phosphonates should be useful in establishing the biological roles of DPP-IV and may have therapeutic utility in preventing organ transplant rejection.

Experimental Section

Synthesis. Benzyl carbamate, diphenyl phosphite, pyrrolidine, piperidine, DCC, and all common chemicals were obtained from Aldrich Co., Milwaukee, WI. (Benzyloxycarbonyl)proline (Z-Pro) and N^α,N^ϵ-bis(benzyloxycarbonyl)lysine were obtained from Bachem Fine Chemicals, CA. The purity of each new synthesized compound was checked by TLC, ^{1}H NMR, mass spectroscopy (FAB), and elemental analysis. In the case of multistep synthesis, the first and final products were checked by ^{1}H NMR, FAB spectra, and elemental analysis. The solvent system used for TLC was chloroform–acetone (9:1). Preparative thin-layer chromatography was performed with plates precoated with silica gel (Merck). The NMR spectra were recorded on a Varian Gemini 300 MHz instrument in CDCl$_3$, DMSO-d_6, or D$_2$O solutions. Mass spectra (FAB) were recorded on a VG 70-SE mass spectrometer. Elemental analyses were performed by Atlantic Micro-Lab Inc., Norcross, GA.

Bis(4-chlorophenyl) phosphite and bis(4-fluorophenyl) phosphite were prepared from tris(4-chlorophenyl) phosphite and tris(4-fluorophenyl) phosphite, respectively, using a previously described procedure.[27] Tris(4-chlorophenyl) phosphite was prepared from 4-chlorophenol and phosphorus trichloride with 3 equiv of triethylamine as a base using a modification of a previous procedure.[28] Similarly, tris(4-fluorophenyl) phosphite was prepared from 4-fluorophenol and phosphorus trichloride using 1 equiv of triethylamine as a base.

Diphenyl Pyrrolidine-2-phosphonate Hydrochloride (HCl·Pro^P(OPh)$_2$, 1). This compound was synthesized from

ester.[20] A mixture of 1-pyrroline trimer (17 mmol, 3.5 g) and diphenyl phosphite (50 mmol, 11.7 g) was heated at 85 °C for 1.5 h under argon to give crude diphenyl pyrrolidine-2-phosphonate which was dissolved in 100 mL of dry diethyl ether, filtered, and saturated with dry gaseous HCl. The precipitated hydrochloride **1** was collected by filtration, washed with ether, and recrystallized from acetone to give the pure product as a white solid in 49% yield: mp 146–148 °C; ^{1}H-NMR (D$_2$O) δ (ppm) 2.0–2.5 (m, 4H), 3.40 (m, 2H), 4.25 (m, 1H), 6.90–7.30 (m, 10H); MS (FAB) *m/e* (rel intensity) 304 (M − Cl)$^+$ (100). Anal. (C$_{16}$H$_{19}$NO$_3$PCl): C, H, N, Cl.

Diphenyl Piperidine-2-phosphonate Hydrochloride (HCl·Pip^P(OPh)$_2$, 2). This compound was prepared from the trimer of 2,3,4,5-tetrahydropyridine[30] and diphenyl phosphite using the procedure previously described for the synthesis of the diethyl ester.[21] A mixture of the trimer (10 mmol, 2.5 g) and diphenyl phosphite (30 mmol, 7.0 g) was heated for 1.5 h at 100 °C under argon. The resulted crude diphenyl piperidine-2-phosphonate was dissolved in 100 mL of dry ether, undisolved material was removed by filtration, and the solution was saturated with gaseous HCl. The precipitated hydrochloride **2** was collected by filtration, washed with ether, dried, and recrystallized from acetone to give a white solid in 41% yield: mp 172–174 °C; ^{1}H-NMR (D$_2$O), δ (ppm) 1.5–2.4 (m, 6H), 3.05 (m, 1H), 3.45 (m, 1H), 4.10 (m, 1H), 6.9–7.4 (m, 10H); MS (FAB) *m/e* (rel intensity) 318 (M − Cl)$^+$ (100). Anal. (C$_{17}$H$_{21}$NO$_3$PCl): C, H, N, Cl.

Bis(4-chlorophenyl) Pyrrolidine-2-phosphonate Hydrochloride (HCl·Pro^P(OPh-4-Cl)$_2$, 3). A mixture of 1-pyrroline trimer and bis(4-chlorophenyl) phosphite was reacted using the procedure described for compound **1**. Hydrochloride **3** was obtained by dissolving the crude phosphonate in ether saturated with gaseous HCl (white solid in 30% yield): mp 160–165 °C dec; ^{1}H-NMR (D$_2$O) δ (ppm) 1.8–2.5 (m, 4H), 3.4 (m, 2H), 4.30 (m, 1H), 7.0 (m, 4H), 7.2 (m, 4H); MS (FAB) *m/e* (rel intensity) 372 (M − Cl)$^+$ (100). Anal. (C$_{16}$H$_{17}$NO$_3$PCl$_3$): C, H, N.

Bis(4-chlorophenyl) Piperidine-2-phosphonate Hydrochloride (HCl·Pip^P(OPh-4-Cl)$_2$, 4). The trimer of 2,3,4,5-tetrahydropyridine and bis(4-chlorophenyl) phosphite were reacted using the procedure described for compound **2**. The phosphonate hydrochloride **4** was obtained by reaction with HCl as described above in 55% yield: mp >140 °C dec; ^{1}H-NMR (D$_2$O) δ (ppm) 1.3–2.1 (m, 6H), 3.2–3.5 (m, 2H), 4.0 (m, 1H), 6.86 (m, 4H), 7.1 (m, 4H); MS (FAB) *m/e* (rel intensity) 386 (M − Cl)$^+$ (70). HRMS calcd for C$_{17}$H$_{19}$N$_1$O$_3$P$_1$Cl$_2$ *m/e* 386.0479, found 386.0468.

Bis(4-fluorophenyl) Piperidine-2-phosphonate Hydrochloride (HCl·Pip^P(OPh-4-F)$_2$, 5). A mixture of piperidine trimer (4.5 g, 54 mmol) and bis(4-fluorophenyl) phosphite (14.8 g, 55 mmol) was heated at 90–100 °C for 3 h under nitrogen. The resulted oil was cooled and dissolved in a mixture of 50 mL of CH$_2$Cl$_2$ and 50 mL of ether. The solution was saturated with dry HCl, and the oil was separated and solidified after several hours. The solid was filtered, washed with ether, and dried. The hygroscopic material was stirred in 200 mL of dry ether for several hours, and the yellowish solid was filtered and dried. The product was obtained in 54% yield (11.6 g) and used for subsequent reaction: mp 155–165 °C dec; ^{1}H NMR (D$_2$O) δ 7.2–6.8 (m, 8H), 3.6–2.9 (m, 3H), 2.2–1.4 (m, 6H); MS (FAB) *m/e* (rel intensity) 354 (M − Cl)$^+$ (100); HRMS calcd for C$_{17}$H$_{19}$N$_1$O$_3$P$_1$F$_2$ *m/e* 354.1070, found 354.1098.

Dipeptide Synthesis: General Procedure. The hydrochloride of the phosphonates (**1**, **2**, **3**, **4** or **5**) (5 mmol) and triethylamine (5 mmol) were dissolved in 25 mL of CH$_2$Cl$_2$ and cooled to −10 °C. A Z-blocked amino acid (5 mmol) was added, and the mixture was stirred at −10 °C for 15 min. The coupling reagent DCC (6 mmol) in 25 mL of CH$_2$Cl$_2$ was added, and the mixture was stirred at −10 °C for 2 h and 20 h at room temperature. The DCU precipitate was removed by filtration, and the filtrate was evaporated. The residue was dissolved in 100 mL of ethyl acetate and filtered. The organic layer was washed subsequently with 50 mL of 1 M HCl, water, 6% NaHCO$_3$, and water and dried over MgSO$_4$. The filtrate

25 mL of ether. The dipeptide was dried *in vacuo* and recrystallized from hexane−ether.

The Z group of the dipeptides were removed by hydrogenolysis or treatment with 30% HBr in acetic acid. The Z-blocked dipeptide (1−2 mmol) was dissolved in 100 mL of methanol, 1 equiv of concentrated hydrochloric acid (1−2 mmol) and 5% Pd on carbon (0.5−1.0 g) were added, and the mixture was hydrogenated at room temperature for 2−3 h. After hydrogenation, the catalyst was removed and the filtrate was evaporated to give the deblocked dipeptide hydrochloride, which was recrystallized from methanol−ether or ether. The Z-blocked dipeptide (1 mmol) can also be treated with 1 mL of 30% HBr/AcOH and stirred at room temperature for 1 h. The mixture was protected against moisture during stirring. The solution was diluted with 50 mL of dry ether and kept at 0 °C for 1−2 h. The hydrobromide of the dipeptide precipitated and was filtered, washed with dry ether, and dried as a yellow-brown solid.

Diphenyl Alanylpyrrolidine-2-phosphonate Hydrochloride (HCl·Ala-Pro^P(OPh)$_2$, 6). Z-Ala-Pro^P(OPh)$_2$ was obtained as a thick oil in 90% yield: ^{1}H-NMR (CDCl$_3$) δ (ppm) 1.3 (dd, 3H), 1.5−2.5 (m, 5H), 3.3−3.8 (m, 2H), 4.1 (m, 1H), 4.5 (m, 1H), 5.10 (m, 2H), 5.7 (dd, 1H), 7.0−7.4 (m, 15H). Hydrogenolysis of Z-Ala-Pro^P(OPh)$_2$ gave the product **6** as a hygroscopic solid in 65% yield: mp 80−85 °C; MS (FAB) m/e (rel intensity) 375 (M − Cl)$^+$ (100). The product was further purified by silica gel column chromatography eluted with CHCl$_3$:MeOH:CH$_3$COOH, 8:2:0.1: ^{1}H-NMR (DMSO) δ (ppm) 1.20−1.35 (d, 3H, J = 6.8 Hz), 1.9−2.4 (m, 4H), 3.6−3.75 (m, 2H), 3.9−4.05 (m, 1H), 4.8−4.9 (m, 1H), 7.05−7.5 (m, 10H); ^{31}P-NMR (DMSO, ppm), 19.2433, 19.1841 (1:0.5); MS (FAB) m/e (rel intensity) 375 (M − CH$_3$COO)$^+$ (100); HRMS calcd for C$_{19}$H$_{24}$N$_2$O$_4$P$_1$ m/e 375.1474, found 375.1473.

Diphenyl Alanylpiperidine-2-phosphonate Acetate (CH$_3$COOH·Ala-Pip^P(OPh)$_2$, 7). Z-Ala-Pip^P(OPh)$_2$ was obtained as a thick oil in 76% yield: ^{1}H-NMR (CDCl$_3$) δ (ppm) 1.2−1.3 (dd, 3H), 1.5−2.4 (m, 6H), 3.6−3.8 (m, 2H), 4.5 (m, 1H), 5.10 (s, 2H), 5.6 (m, 1H), 5.8 (dd, 1H), 7.0−7.4 (m, 15H). Hydrogenolysis of Z-Ala-Pip^P(OPh)$_2$ using 1 equiv of acetic acid gave the product **7** as a glass-like solid in 82% yield: mp 60−70 °C; MS (FAB) m/e (rel intensity) 389 (M − CH$_3$COO)$^+$ (100). The product was further purified by silica gel column chromatography eluted with CHCl$_3$:MeOH:CH$_3$COOH, 8:2:0.1: ^{1}H-NMR (DMSO) δ (ppm) 1.0 (d, 3H, J = 6.5 Hz), 1.4−2.3 (m, 6H), 1.9 (s, 3H), 3.4−3.5 (t, 2H, J = 13 Hz), 3.8−4.0 (m, 2H), 5.45−5.50 (m, 1H), 7.0−7.4 (m, 10H); ^{31}P-NMR (DMSO, ppm) 18.9337, 18.7177 (1:0.1); HRMS calcd for C$_{20}$H$_{26}$N$_2$O$_4$P$_1$ m/e 389.1630, found 389.1639.

Monophenyl Alanylpiperidine-2-phosphonate Acetate (CH$_3$COOH·Ala-Pip^P(OH)(OPh), 8). A small amount of the monophenyl ester **8** was isolated in 10% yield during the workup of product **7** as a white solid: mp 175−180 °C dec; MS (FAB) m/e (rel intensity) 313 (M − CH$_3$COO)$^+$ (100). The product was further purified by silica gel column chromatography eluted with CHCl$_3$:MeOH:CH$_3$COOH, 8:2:0.1: ^{1}H-NMR (DMSO) δ (ppm) 1.1−1.3 (d, 3H, J = 6.5 Hz), 1.4−2.2 (m, 6H), 1.9 (s, 3H), 3.1−3.2 (t, 1H, J = 13 Hz), 4.1−4.2 (m, 1H), 4.25−4.42 (m, 2H), 6.9−7.3 (m, 5H); ^{31}P-NMR (DMSO, ppm) 13.2251, 13.1494 (1:0.5); HRMS calcd for C$_{14}$H$_{22}$N$_2$O$_4$P$_1$ m/e 313.1317, found 313.1314.

Diphenyl Phenylalanylpyrrolidine-2-phosphonate Hydrobromide (HBr·Phe-Pro^P(OPh)$_2$, 9). Z-Phe-Pro^P(OPh)$_2$ was obtained as a thick oil which partially solidified in 61% yield: ^{1}H-NMR (CDCl$_3$) δ (ppm) 1.0−2.0 (m, 4H), 3.25 (d, 2H), 3.0−3.5 (m, 3H), 4.7 (m, 1H), 5.05 (m, 1H), 5.15 (s, 2H), 5.35 (m, 1H), 7.0−7.4 (m, 20H). Deblocking of Z-Phe-Pro^P(OPh)$_2$ with 30% HBr/AcOH gave the product **9** as a yellow-brown hygroscopic solid in 33% yield: mp >140 °C dec; ^{1}H-NMR (D$_2$O) δ 1.0−2.0 (m, 4H), 3.0−3.4 (m, 3H), 3.3 (d, 2H), 4.3 (m, 1H), 4.7 (m, 1H), 7.0−7.4 (m, 15H); MS (FAB) m/e 451 (M − Br)$^+$ (100); HRMS calcd for C$_{25}$H$_{28}$N$_2$O$_4$P$_1$ m/e 451.1786, found 451.1769.

Diphenyl Lysylpyrrolidine-2-phosphonate Dihydrobromide (2HBr·Lys-Pro^P(OPh)$_2$, 10). The lysine derivative

(ppm) 1.0−2.5 (m, 8H), 3.0−4.0 (m, 4H), 4.5 (m, 1H), 4.9 (m, 1H), 5.1 (2s, 4H), 5.5−5.8 (dd, 2H), 7.0−7.4 (m, 20H). Treatment of Z-Lys(Z)-Pro^P(OPh)$_2$ with 30% HBr/AcOH gave the product **10** as a yellow brown hygroscopic solid in 64% yield: mp >85 °C dec; ^{1}H-NMR (D$_2$O) δ (ppm) 1.2−2.5 (m, 8H), 2.8−3.0 (m, 4H), 3.55 (t, 3H), 4.1−4.5 (m, 2H), 7.0−7.5 (m, 10H); MS (FAB) m/e (rel intensity) 432 (M − H − 2Br)$^+$ (100); HRMS calcd for C$_{22}$H$_{31}$N$_3$O$_4$P$_1$ m/e 432.2052, found 432.2059.

Diphenyl Lysylpiperidine-2-phosphonate Dihydrochloride (2HCl·Lys-Pip^P(OPh)$_2$, 11). The lysine derivative Z-Lys(Z)-OH was reacted with phosphonate **2** to give diphenyl Z-Lys(Z)-Pip^P(OPh)$_2$ as a thick oil in 65% yield: ^{1}H-NMR (CDCl$_3$) δ (ppm) 1.0−2.0 (m, 12H), 3.0−3.2 (m, 2H), 3.4−3.8 (m, 2H), 4.4−4.8 (m, 1H), 5.1 (2s, 4H), 5.5−5.9 (m, 2H), 7.0−7.5 (m, 20H). Hydrogenolysis of Z-Lys(Z)-Pip^P(OPh)$_2$ gave the product **11** as a white solid in 54% yield: mp 110−115 °C dec; ^{1}H-NMR (D$_2$O) δ (ppm) 1.3−2.1 (m, 12H), 2.8−3.0 (m, 2H), 3.3−3.7 (m, 2H), 4.3−4.5 (m, 1H), 7.0−7.4 (m, 10H); MS (FAB) m/e (rel intensity) 446 (M − H − 2Cl)$^+$ (100); HRMS calcd for C$_{23}$H$_{33}$N$_3$O$_4$P$_1$ m/e 446.2208, found 446.2213.

Bis(4-chlorophenyl) Alanylpyrrolidine-2-phosphonate Hydrochloride (HCl·Ala-Pro^P(OPh-4-Cl)$_2$, 12). (Benzyloxycarbonyl)alanine (Z-Ala) was reacted with phosphonate **3** to give Z-Ala-Pro^P(OPh-4-Cl)$_2$ (**12a**) as a semisolid in 41% yield: ^{1}H-NMR (CDCl$_3$) δ (ppm) 1.25−1.35 (dd, 3H), 1.8−2.5 (m, 4H), 3.3−3.9 (m, 2H), 4.55 (m, 1H), 5.0 (m, 1H), 5.1 (s, 2H), 5.5−5.7 (dd, 1H), 7.0−7.4 (m, 13H); MS (FAB) m/e (rel intensity) 577 (M + 1)$^+$ (100). Hydrogenolysis of Z-Ala-Pro^P-(OPh-4-Cl)$_2$ gave the product **12** as a hygroscopic white solid in 53% yield: mp 88−91 °C; MS (FAB) m/e (rel intensity) 443 (M − Cl)$^+$ (100). The product was further purified by silica gel column chromatography eluted with CHCl$_3$:MeOH:CH$_3$COOH, 8:2:0.1: ^{1}H-NMR (DMSO) δ (ppm) 1.25−1.35 (d, 3H, J = 7.1 Hz), 2.0−2.4 (m, 4H), 3.6−3.8 (m, 2H), 4.1−4.3 (m, 1H), 4.8−4.9 (m, 1H), 7.2−7.5 (m, 8H); ^{31}P-NMR (DMSO, ppm), 19.8748, 19.6918 (1:0.33); MS (FAB) m/e 443 (M − CH$_3$COO)$^+$; HRMS calcd for C$_{19}$H$_{22}$N$_2$O$_4$Cl$_2$P$_1$ m/e 443.0694, found 443.0693.

Bis(4-chlorophenyl) Alanylpiperidine-2-phosphonate Hydrochloride (HCl·Ala-Pip^P(OPh-4-Cl)$_2$, 13). (Benzyloxycarbonyl)alanine (Z-Ala) was reacted with phosphonate **4** to give Z-Ala-Pip^P(OPh-4-Cl)$_2$ (**13a**) as a thick oil in 38% yield; the compound solidified when methanol was added: mp 156−162 °C; ^{1}H-NMR (CDCl$_3$) δ (ppm) 1.15−1.25 (2d, 3H, J = 7.6 Hz), 1.4−2.3 (m, 6H), 3.65−3.85 (m, 2H), 4.7−4.8 (m, 1H), 5.1 (s, 2H), 5.5−5.65 (m, 1H), 5.8 (d, 1H), 7.0−7.4 (m, 8H), 7.35 (s, 5H); ^{31}P-NMR (CDCl$_3$, ppm), 17.7472; MS (FAB) m/e (rel intensity) 591 (M + 1)$^+$ (100). Anal. (C$_{28}$H$_{29}$N$_2$O$_6$PCl$_2$): C, H, N, Cl. Hydrogenolysis of Z-Ala-Pip^P(OP-4-Cl)$_2$ gave **13** as a hygroscopic solid in 49% yield: mp 110−115 °C dec; MS (FAB) m/e (rel intensity) 457 (M − Cl)$^+$ (100). The product was further purified by preparative thin-layer chromatography eluted with CHCl$_3$:MeOH:CH$_3$COOH, 8:2:0.1 ^{1}H-NMR (DMSO) δ (ppm) 1.0−1.2 (d, 3H, J = 7.4 Hz), 1.4−2.3 (m, 6H), 3.4−3.5 (m, 1H), 3.9−4.05 (m, 2H), 5.45−5.55 (m, 1H), 7.1−7.5 (m, 8H); ^{31}P-NMR (DMSO, ppm) 19.5526; MS (FAB) m/e (rel intensity) 457 (M − CH$_3$COO)$^+$ (100); HRMS calcd for C$_{20}$H$_{24}$N$_2$O$_4$Cl$_2$P$_1$ m/e 457.0851, found 457.0834.

Bis(4-fluorophenyl) Alanylpiperidine-2-phosphonate Hydrochloride (HCl·Ala-Pip^P(OPh-4-F)$_2$, 14). (Benzyloxycarbonyl)alanine (Z-Ala) was reacted with phosphonate **5** to give Z-Ala-Pip^P(OPh-4-F)$_2$ (**14a**) as a thick oil in 25% yield: TLC (CHCl$_3$:acetone, 9:1) R_f = 0.45; ^{1}H NMR (DMSO) δ 7.35 (s, 5H), 7.4−6.9 (m, 8H), 5.4−5.1 (m, 1H), 5.0 (s, 2H), 4.7−4.3 (m, 1H), 3.9 (m, 1H), 3.5−3.1 (m, 2H), 2.0−1.1 (m, 6H), 1.25 (dd, 3H); MS (FAB) m/e 559 (M + H)$^+$. The hydrogenolysis of **14a** (1.3 g, 2.3 mmol) in methanol in the presence of 1 equiv of HCl and 5% Pd/C gave a hygroscopic solid which was recrystallized from methanol and ether in 57% yield (0.6 g): mp 105−115 °C dec; MS (FAB) m/e 425 (M − Cl)$^+$. The product was further purified by silica gel column chromatography eluted with CHCl$_3$:MeOH:CH$_3$COOH, 8:2:0.1: ^{1}H NMR (DMSO) δ (ppm) 1.2−1.4 (2d, 3H, J = 8.4 Hz), 1.4−22 (m, 6H), 3.8−3.95 (m, 1H), 4.4−4.5 (m, 2H), 5.35−5.45 (m, 1H), 7.15−7.3 (m, 8H); ^{31}P-NMR (DMSO, ppm) 19.4758, 19.3616 (0.12:1); MS

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